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Starch Based Active Packaging Film Reinforced With Empty Fruit Bunch (EFB) Cellulose Nanofiber

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Abstract

Biopolymer active packaging is known to have low mechanical strength and highly brittle. Regardless to its disadvantage, polymers from natural sources have attracted serious attention since the non-renewable sources for example petroleum, the major precursor of plastic manufacturing become depleted. Starch-Chitosan for instance is a hybrid film that entirely green as it produced from a renewable material and totally degradable. The addition of chitosan in film packaging able to kill pathogen hence increases the food shelf life. Through nanotechnology advance, nanomaterial can be used for material reinforcement. Nowadays, greener approach could be applied by incorporating natural cellulose nanofiber into the film matrix. Oil palm empty fruit bunch (OPEFB) fiber that rich of cellulose contents could be treated chemically to purify the cellulose in the fiber. Cellulose fiber obtained was cut to a nano-size using acid hydrolysis. Transmission Electron Microscopy (T.E.M) obtained shown the nanofiber size was ranged between 1-100nm in diameter. Nanocomposite film formulation, was constructed by varying the cellulose nanofiber incorporation between 2-10% per weight of starch. The strength of the films was measured as well as antimicrobial properties. The addition of 2% cellulose nanofiber into the film matrix exhibits high tensile strength with 5.25Mpa compared to starch-chitosan hybrid film with 3.96Mpa. However, no significant improvement in tensile strength was distinguished beyond that ratio. Antimicrobial analysis shows that the addition of cellulose nanofiber could increase the inhibition effect towards gram-positive bacteria but not towards gram-negative bacteria. The addition of 2% cellulose nanofiber increased the inhibition diameter towards gram positive bacteria, *Bacillus subtilis* up to 33%. However, inhibition towards *Bacillus subtilis* decreased with the incorporation of more cellulose nanofiber. In gram-negative bacteria *Escherichia coli*, the addition of cellulose nanofiber does not give significant effect to bacterial. In General, the addition of the unique structure of cellulose nanofiber in the starch based polymer system could enhance the mechanical strength of the film and increase the inhibition of the gram positive bacteria.

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Nomenclature

OPEFB	Oil Palm Empty Fruit Bunch
%T	Percentage of transmittance
CNF	Cellulose nanofiber
O. D	Optical Density
S:C	Starch:Chitosan
λ	Wavelength
MPa	*Mega* pascal

1. Introduction

Reinforcement of biopolymer using natural filler such as fiber has attracted consideration as it applied greener way for material development. It's able to create an end product that environment friendly by using renewable source for both matrix and filler. The renewable source of raw material may come from agricultural residues from different parts of plants. Natural fibers are naturally-occurring composite consisting mainly of cellulose fibrils embedded in lignin matrix[5]. Cellulose on the other hand is the abundant organic compound that generally a structural component of the cell walls of many plants. It is present mainly in the forest with wood as the most important source. Generally, cellulose fiber has unique pecking order. It comprised of nanofibers assemblies with diameter that range from 2 – 20 mm and a length of more than few micrometers[1]. Cellulose is found to be the most common organic polymer and considered as an almost infinite source of raw material for the increasing demand in environment-friendliness and biocompatibility. As lignocellulosic fiber becomes vital, many possible plants were tested. Natural fiber not only can be harvested from cotton, wool and other ancient source, but it covers different variety and different source all over the world and oil palm empty fruit bunch is one of them. The combination of those fibers with natural or synthetic polymer is expected to produce material with great properties. As reported in previous research, the nano-biopolymer composite was developed to enhance native biopolymer properties mechanically and physically. Fiber reinforced biopolymer was actually being developed before, however the application was limited for rigid biopolymer that applied in solid packaging. The application of fiber in a thin film that classified as flexible packaging is however not really suitable. The nano-scale fiber was then evolved by "cutting off" the micro structure of the fiber into nano-size. Previous report shows that the nano-sized fiber exhibits unique characteristics that could give better reinforcement to the film[2]. Hence, there are many research has been conducted in making nano-size fiber, for example chemo-mechanical process using mechanical stirring and strong acid. An an outcome, the incorporation of this new-formed fiber into thin film were found to be more versatile to any types of composite especially in thin film polymer. The nanofiber was found to be appropriate filler for thin film packaging because it was not strongly affect the appearance of the film compared to micro size fiber which creates lumpy structure deposited on the surface of the film. The incorporation of nano-sized fiber also increases the matrix-filler interaction that may contribute the mechanical reinforcement of the film.

2. Experimental

2.1. Materials

Oil palm empty fruit bunch (OPEFB) fiber was donated by Universiti Kebangsaan Malaysia - Malaysia Palm Oil Board (UKM-MPOB) research center, Bangi, Malaysia. The fiber provided was screw pressed to remove excess oil and moisture. It was compressed into a cube-shaped, stacked, and sealed until further use.

2.2. Cellulose nanofiber preparation

The extraction of oil palm empty fruit bunch (OPEFB) cellulose nanofiber involved two steps. It includes pretreatment/bleaching and hydrolysis process.

2.2.1. Pretreatment and bleaching process

In order to produce high purity of cellulose fiber, pretreatment and bleaching process are required to remove hemicelluloses, lignin and other extractives such as waxes and ashes. Firstly, the fiber that collected from residue waste of empty fruit bunch was undergone the press - screw process, a common process in palm oil industry to remove excess oil and moisture. The fiber then shredded by hammer mill process and let to dry in the oven at temperature 70ºC for 1 day. The fiber then was cut using the crusher machine, and sieved to get uniform sizes (0.1-1mm) and also to remove ashes. 100gm fibers were immersed in hot distilled water at temperature 80ºC for at least 12 hours to eliminate impurities and huge particles[12]. The water will be removed from the EFB fiber by filtration. The process was continued according to the pretreatment method that developed by Fazlena *et. al*. (2011)[4]. The fiber was added in 1000 ml of 2.5 Mol L^{-1} Sodium hydroxide and autoclaved for 15 min at 121 °C. The EFB fiber will be separated from the sodium hydroxide solution using vacuum filtration with no 1 Whatman filter paper. EFB fibre will go through a bleaching process using sodium hypochlorite solution with 6-14% active chlorine until the pH of the fibre became 3.0. The process was repeated for three times until white fiber obtained. Next, the fibre was washed sufficiently with distilled water until the pH of the fibre became 7.0. The treated fiber will be dry in 37°C and stored for further use.

2.2.2. Hydrolysis of the cellulose fiber

Nano-size cellulose fiber can be achieved by the hydrolysis process using concentrated acid. 10 grams of EFB fibers were mixed into 64 what% of 105 ml H_2SO_4 solution as method developed by Fahma et al., (2010)[3]. It was stirred vigorously using a magnetic stirrer at 45 °C for 90 min. 400ml of cold water was added to stop the hydrolysis process. The suspension was obtained by centrifuge the suspension in 11,000 RPM for 10 min. The precipitate was washed repeatedly by adding deionized water, vigorously agitated and centrifuged. The process was repeated until the pH of the suspension reached 5. The dialysis of the fiber suspension was carried out for 5 days until the pH became constant. The cellulose nanofiber suspension was homogenized using ultra turrax T-25 for 30 seconds and stored in a refrigerator before further use.

2.3. Preparation of cellulose nanofiber reinforced starch based active packaging

The control active film was prepared without the incorporation of cellulose nanofiber. 5 grams of wheat starch, 2.5 grams of glycerol, and 100 ml of distilled water was blended together by magnetic stirrer bar with medium stirring. The antimicrobial agent, chitosan that previously dissolve into distilled water contained 10% of acetic acid was added into the mixture. Heat was applied gradually while homogenizing the mixture. The heat was kept between 85-90°C and continued for another 30 minutes. The film was cast by pipetting 10ml of the mixture onto a petri dish, then let to dry at temperatures of 50-60°C for 24 hours. The film was then peeled off from a petri dish. Cellulose nanofiber reinforced active packaging film was prepared by adding previous mixture with different ratios of nanocellulose loading; 2-10% of cellulose nanofiber (w/w) to total weight of starch. The thickness of the film was measured by micrometer IP 65 (Mitutoyo Manufacturing, Tokyo, Japan) to the nearest 0.001 mm, at 10 random positions on the film for each samples designed for tensile tests. The film transparency was obtained by UV-vis spectroscopy. Film samples were cut to 1 × 3 cm and placed on the internal side of a spectrophotometer cell. The reading was recorded using the transmittance mode at wavelength 600nm and film transparency was defined as the percentage of light that allowed to be transmitted across the film. Percentage of transmittance (%T) was calculated by beer-lambert law as stated below, where A=absorbance at wavelength 600nm.

$$\%T = 100\ (10^{\wedge -A}) \qquad (1)$$

2.4 Mechanical properties of cellulose nanofiber reinforced film

The mechanical strength such as tensile strength (TS), % elongation (E) and young modulus (Y) values of the films are investigated using tensile machine-Lloyd LRX materials testing machine (Lloyd Instruments Ltd, Fareham, UK), referring to standard method ASTM D 638-03. The gauge length was set to 9.6 mm and the crosshead speed 10 mm/min. The film was cut into strips in dumbbell. The tests were carried out at 23 ± 2ºC and 50 ± 5% RH. Total average of 5 specimens was taken for each determination. Each determination was taken from an average of five specimens. Then, the mechanical strength was compared among 0%, 2%, 4% 6%, 8% and 10% addition of cellulose nanofiber incorporation.

2.5 Antimicrobial properties of formed film

Antimicrobial activity of formed film was obtained by the agar diffusion method and liquid culture test. The agar diffusion test was intended to impersonate the antibacterial activities of the film on the solid food surface and liquid culture test on the other hand infers the antibacterial activities of the film on liquid-form food.

2.5.1 Agar Diffusion Method.

The antimicrobial activity of the film towards bacteria was carried out using the agar diffusion test. The agar was prepared by mixing 8 grams of Luria Agar Base, Miller with 1L of water and autoclaved before pouring onto the petri dish. The zone of inhibition assay on solid media was applied for determination of the antibacterial effects of films against Escherichia coli, and Bacillus subtilis that represent gram-positive and gram-negative respectively. 0.1 ml of testing bacteria inoculums solution was poured and spread onto the agar. Using a puncher (D=1 cm), each sample was cut and placed on the agar surface that contain bacteria. The plates were incubated in the oven for 24 h at temperature of 37ºC. The diameter of inhibition zone was measured using a ruler by three different points.

2.5.2 Enumeration (Liquid Culture Test)

The other antimicrobial activity of the film was carried on using liquid culture test. The test simulates the antimicrobial activity and the characteristics of the active film in liquid. Each film was cut into squares (2cm x 2cm) and immersed in 25ml of universal bottle containing 19.9 ml of standard Luria Broth, Miller. 100 µl of *Eschericha coli/ Bacillus. Subtilis* (late exponential phase) was added into the universal bottle. The bottles contain broth and inoculums were incubated in an incubator orbital shaker at 200rpm and temperature of 37°C. The growth of the bacteria was determined by measuring the optical density of the broth. The reading was sampled at time 0, 2^{nd}, 4^{th}, 8^{th}, 12nd and $24^{th}$and the wavelength λ = 600nm was set for each determination.

3. Results and discussion

3.1 Cellulose nanofiber preparation

Pretreatment and bleaching process produce purer and whiter fiber strand. The pretreatment process using concentrated sodium hydroxide with high pressure and temperature are able to exploded the fiber so that hemicelluloses and lignin could be removed effectively[4]. The process was called steam explosion techniques that originally developed by W. H. Mason in 1928. A different source of material needs different steam explosion temperature and time of retention. One material might need more retention time and higher temperature than the other. However, previous researchers have confirmed that steam explosion could separate hemicelluloses and lignin effectively which greatly enhance the subsequent process after the pretreatment process[13]. Table 1 shows the composition analysis of cellulose, lignin , hemicelluloses and other extractives using the Technical Association of the Pulp and Paper Industry (TAPPI) method which confirmed large fraction of hemicelluloses and lignin was removed during the pretreatment process. The composition of hemicelluloses and lignin was extensively removed

by acid hydrolysis of cellulose nanofiber leaving a higher percentage of cellulose content in the final product. During the pretreatment, hemicelluloses and lignin was removed up to 80% and 89% respectively whilst hydrolysis process was subsequently removed that produce purer cellulose.

Table 1 : Composition (%) of oil palm empty fruit bunch fiber for untreated, treated and acid-hydrolyzed

Component (%)	Untreated EFB Fiber	Pre-treated EFB Fiber	Cellulose Nanofiber
Cellulose	41 ± 2	90 ± 1	92±2
Hemicellulose	22 ± 3	4.4± 1	3.5±-0.5
Lignin	19 ± 1	2± 0.5	1.5±0.1
Extractives (Ash, waxes, pectin)	2.0 ± 0.3	0.5 ± 0.2	0.5±0.1

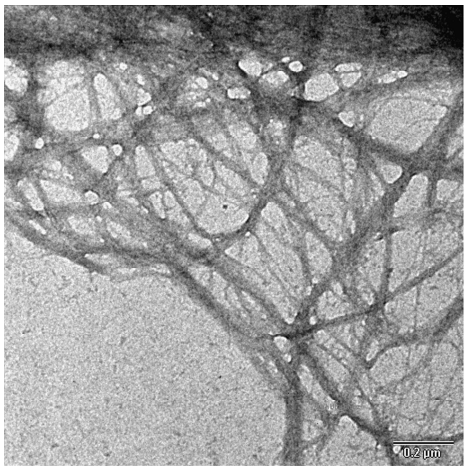

Fig 1: Transmission electron microscopy (T.E.M) x10k Magnification of cellulose nanofibers.

Figure 1 shown the transmission electron microscopy (T.E.M), illustrate the morphology of the fiber after acid hydrolysis process. The fiber that has undergone an acid hydrolysis process was broken down to nano-size diameter ranged between 1-100nm. The strong acid cleaved the stack of cellulose microfibrils and produced the fiber with nano-sized diameter[9]. The amorphous region of microfibrils cleaved by sulfuric acid hydrolysis assisted with mechanical shearing that finally makes the diameter of the fiber to be reduced from microns to nanometers.

3.2 Film making

Figure 2 shows the photograph of the film that was produced from film casting method. Transparent and flexible film was formed. Film transparency is a property to measure the amount of light transmittances across the film with the addition of cellulose nanofiber. Table 2 shows the optical transmittance of the film at wavelength λ=600. The films are transparent at low cellulose nanofiber content but tend to become opaque as the concentration of nanoparticles increases. Based on Yano et al. (2005)[14], reinforcing elements with diameters less than one tenth of visible light wavelengths is not expected to cause light scattering. EFB cellulose nanofiber that was used in this experiment ranged between 1-100nm where the rules only applicable when the cellulose nanofiber diameter is 59nm or less. Significant nanofiber agglomeration may also occur that causing light scattering and finally reduced the light transmittance of the film[14].

Table 2 Absorbance and transparency of the formed films.

Film	Absorbance (A^{600nm})	Percentage of Transmittance %T
0 CNF	0.20	63.10
2%CNF	0.29	51.84
4% CNF	0.35	45.15
6% CNF	0.38	41.24
8% CNF	0.46	34.51
10% CNF	0.50	31.38

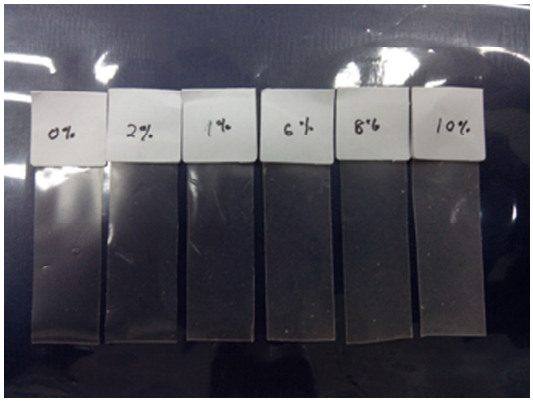

Fig. 2**.** Starch based active packaging film, 0%, 2%, 4%, 6%, 8% and 10% of cellulose nanofiber incorporation**.**

3.3 Mechanical properties of cellulose nanofiber reinforced film

Cellulose nanofiber act as a filler that could improve mechanical properties of the polymer composite. It is possible because the chemical likeness of both filler and matrices provides interfacial adhesion and strong hydrogen bonding[10]. It includes the interfacial adhesion between the nanofiber-nanofiber and nanofiber-matrix interactions. Table 3 shows the mechanical properties include tensile strength, percentage of elongation, and young modulus of the formed film.

Table 3 : Tensile strength, percent elongation and young modulus of formed film

Film	Tensile Stength (TS)	Percent Elongation (E%)	Young Modulus (Y)
Starch + Chitosan	3.96	30.27	358536.26
Starch + Chitosan 2% CNF	5.25	25.71	724357.47
Starch + Chitosan 4% CNF	4.88	27.74	807939.35
Starch + Chitosan 6% CNF	4.80	26.77	710154.16
Starch + Chitosan 8% CNF	5.25	25.71	724357.47
Starch + Chitosan 10%CNF	4.30	25.04	635882.39

2% of EFB cellulose nanofiber incorporation in the film matrix are the optimum ratio that gave most excellent reinforcement capability. The tensile strength was increased to 25% compared to the original film that does not contain cellulose nanofiber. It implied that the addition of 8% of cellulose nanofiber into the film matrix made good interface between matrices and cellulose nanofiber to be reached at this point. Theoretically, in order to reach stable and strong interfacial adhesion, the cellulose nanofiber should be homogeneously dispersed and correctly arranged in the matrix. The addition of more than 8% of cellulose however, decreased the tensile strength. It may because of the agglomeration was occurring due to heterogenous size distribution of cellulose nanofiber. Figure 3 a,

b, c, and d show the Scanning Electron Microscopy (S.E.M) of forming films with the addition of 0%, 2%, 6% and 10% CNF respectively. As the addition of cellulose nanofiber increased, the agglomeration was obviously formed. The agglomeration that occurs subsequently makes the molecules in the film poorly bonded with each other. The cellulose nanofiber also not successfully interfered with starch packing that decreasing intermolecular attraction hence makes the film easy to break and finally reduce the strength of the film.

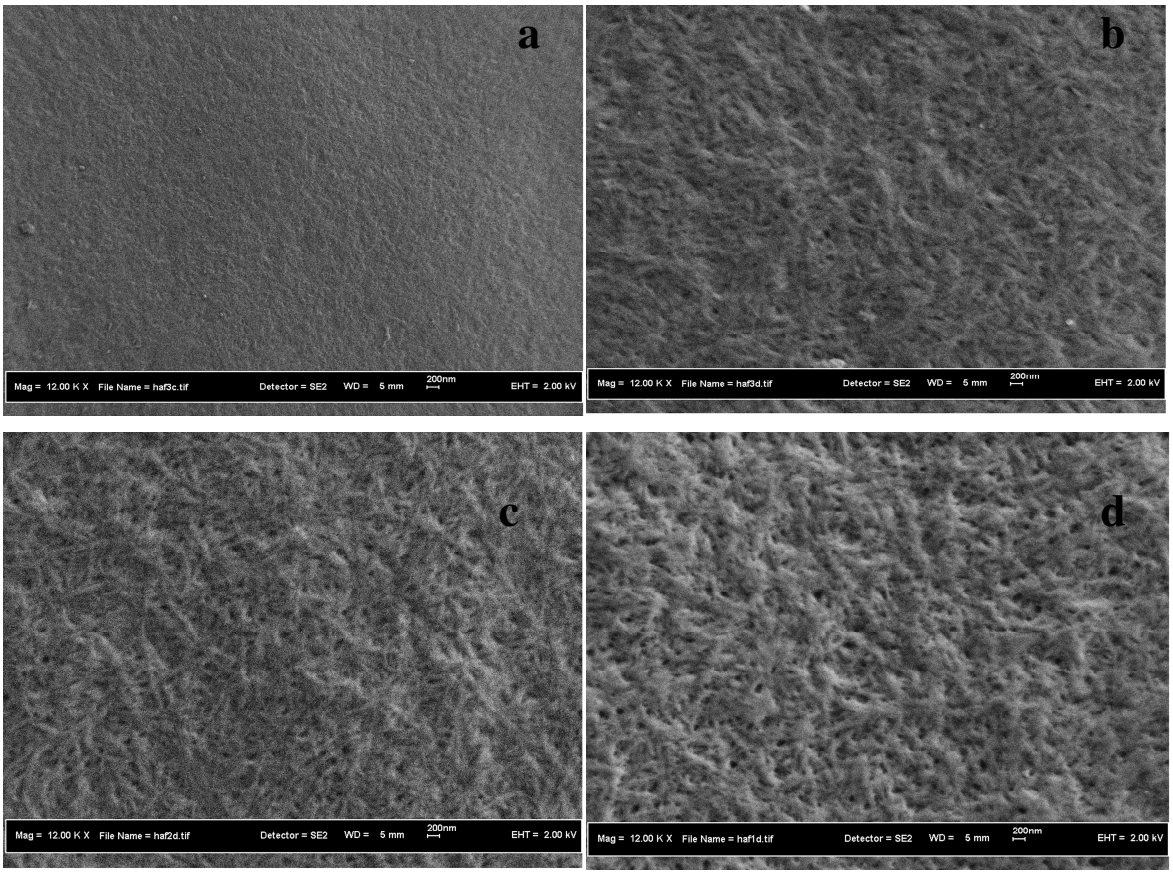

Fig 3 : Field Emission Electron Microscopy (F.E.S.E.M) of Starch-Chitosan hybrid film with incorporation of a) 0% CNF b) 2%CNF c) 6%CNF d) 10%CNF

In general, the successfulness of cellulose nanofiber reinforcement capability is the dispersion and the arrangement of cellulose nanofiber in the matrix. Usually, sufficient amount of nano cellulose in the film matrix could increase the young modulus, and expense its elongation at break[11]. However in this case, the percent of elongation at break was decreased as the incorporation of cellulose nanofiber increased. It is possible to infer that the dispersion of cellulose nanofiber depends on the proportion of the cellulose nanofiber incorporation. The inhomogeneous dispersion of cellulose nanofiber will make the distribution of stress in the film become distorted hence, weakened the whole structure of the film[6].

3.4 Antimicrobial properties of formed film

3.4.1 Agar diffusion method

In general, all antimicrobial film that contains chitosan was given positive antimicrobial activity. The addition of cellulose nanofiber however was affecting the antimicrobial activity of the film. Referring to figure 4, for gram positive bacteria; i.e. Bacillus subtilis, the addition of 2% of cellulose nanofiber have increased the inhibition diameter, however, the diameter of inhibition was reduced as more of cellulose nanofiber incorporated into the film. Basically from the finding, the incorporation of cellulose nanofiber could enhance the bacterial inhibition.

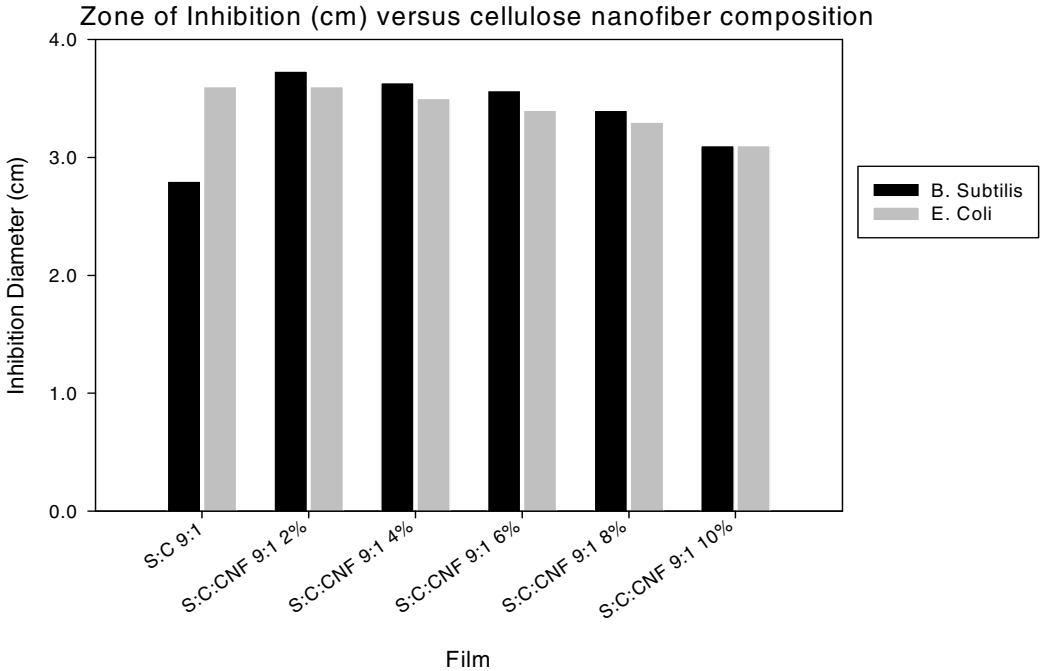

Fig 4**:** The effect of cellulose nanofiber incorporation toward bacteria inhibition of B. subtilis and E. Coli bacteria

Some finding stated the addition of cellulose nanofiber put up uniform dispersion of chitosan particles in the fiber matrix hence, increase the antimicrobial activity[8]. Gram negative bacteria E. coli, however, show that the addition ofcellulose nanofiber didn't improve antimicrobial action of the formed film. The addition of 2% not significantly reduces nor increases the bacteria inhibition. The further addition of cellulose nanofiber however shows the negative effect on bacteria inhibition. This is possible because of good integrity between those three constituents meaning chitosan, cellulose nanofiber and starch might be reached. Generally, interaction between the anionic sulfate groups on nanocellulose and the cationic amine groups from chitosan brings a good interface between the matrix and the filler[7]. Subsequently, the good nanocrystal and polymer interaction provides a composite that not easy to break down, hence reducing the antimicrobial agent release.

3.4.2 Liquid Culture Test

In liquid culture, the behavior of bacteria inhibition should be the same as the agar diffusion method. The higher Optical density value infers that more bacteria live in the culture media. The successful growth pattern of the bacteria in the liquid culture shows that the bacteria are able to replicate productively because of the film that fail to give inhibition towards it and vice versa. In general, starch-chitosan hybrid film is able to give inhibition to both gram positive and gram negative bacteria. Chitosan is positively charged amino group which interacts with

negatively charged microbial cell membranes then leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms. In these findings, the addition of cellulose nanofiber has altered the antimicrobial properties of the film differently according to types of bacterial spectrum. Figure 5 (a) shows the effect of cellulose nanofiber incorporation towards growth pattern of gram-positive bacteria, *Bacillus subtilis*. The addition of 2% of cellulose nanofiber gave the best antimicrobial inhibition compared to other ratios. In fact, the addition of cellulose nanofiber beyond 2% reduces the optical density reading. It's probably cause by the migration of cellulose nanofiber out of the film matrix that interfere with the absorbance reading. The other reason is the structural arrangement of the molecules which comprise starch, chitosan and cellulose nanofiber as mentioned previously. Good integrity between the chitosan, cellulose nanofiber and starch might be reached as the higher percentage of cellulose nanofiber increased hence reducing

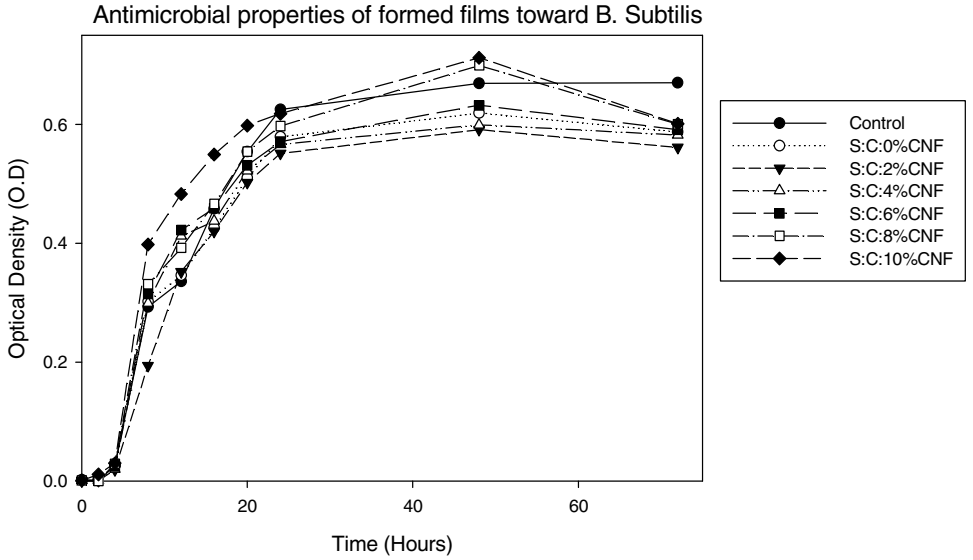

Fig 5 (a) Effect of cellulose nanofiber incorporation towards growth pattern of gram positive bacteria, B. subtilis

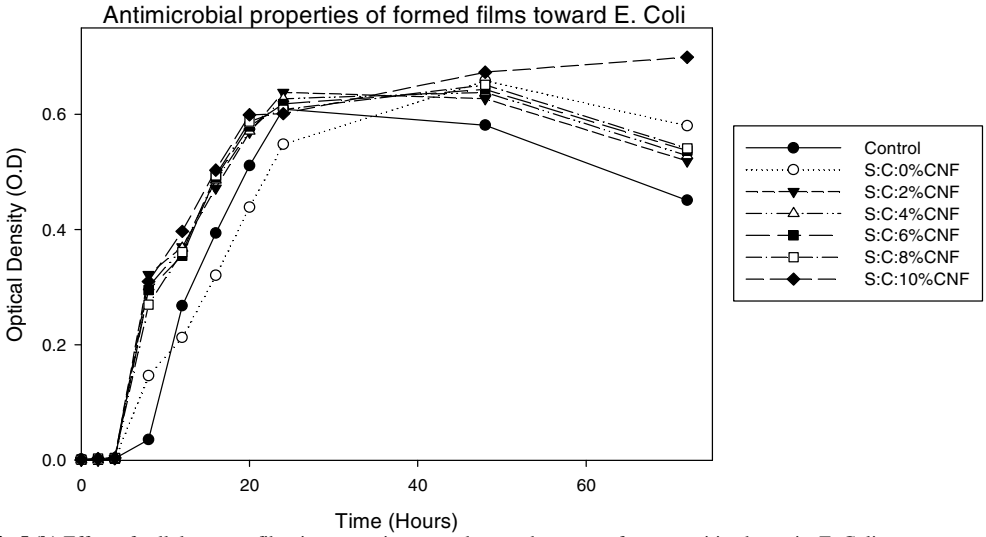

Fig 5 (b) Effect of cellulose nanofiber incorporation towards growth pattern of gram positive bacteria, E. Coli

Figure 5 (b) shows the effect of cellulose nanofiber incorporation towards the growth pattern of gram-negative bacteria, *E. Coli*. In *E. Coli*, the addition of cellulose nanofiber did not give any better inhibition. In contrary, the addition of 2% of cellulose nanofiber shown the increment of absorbance reading and the value keep increasing as more of cellulose nanofiber was incorporated in the film. Oddly, the growth of bacteria in control liquid culture was lower than the cellulose nanofiber incorporated film. The starch-chitosan hybrid film on the other hand gave the lowest bacterial growth. Other than structural integration that not allowed chitosan to be released free, the other reason that causes this result is the bacteria itself. E. coli is gram negative bacteria. The cell wall of Gram-negative bacteria are thick as it also has an outer membrane, which constitutes the outer surface of the wall. A previous study has observed cell structure of the bacteria with the presence of chitosan. It shows the cell wall of gram-positive bacteria were weakened or even broken, while the cytoplasm of Gram-negative bacteria was concentrated and the interstices of the cell were clearly enlarged. This study indicated that the mechanisms of the antimicrobial activity of chitosan were different between gram-positive and gram-negative bacteria. Antimicrobial activity of chitosan towards gram-negative might not as effective compared to gram-positive bacteria because of that reason.

4. Conclusion

Pretreatment process was subjected to remove hemicelluloses, lignin, and other extractives in order to obtain purer cellulose fiber. The cellulose nanofiber from empty fruit bunch was obtained by the hydrolysis process with strong acid. The polymer's strength (i.e., Young's modulus) increased with the addition of cellulose nanofiber. However, the optimum condition for effective reinforcement was detected in 2% of cellulose nanofiber incorporation. The further addition of cellulose nanofiber does not give significant improvements on film strength, elongation at break and Young's modulus. The nature of natural cellulose which asymmetric and nonhomogenous creates unequal dispersion in the film matrices hence lower its mechanical strength[11]. Antimicrobial analysis shows that the addition of cellulose nanofiber could increase the inhibition effect towards gram positive bacteria but not towards gram negative bacteria. However, inhibition towards bacillus subtilis decreased with the incorporation of more cellulose nanofiber. In gram negative bacteria Escherichia coli, the maximum inhibition was increased to 1.94% with the addition of 2% of cellulose nanofiber. The other ratio unfortunately failed to show an increment on inhibition diameter. The optimum inhibition was increased by only 1.94% which is too small, hence, the addition of cellulose nanofiber does not give significant effect of bacterial inhibition. The addition of the unique structure of cellulose nanofiber in the starch based polymer system could enhance the mechanical strength of the film and accelerate the inhibition of the gram positive bacteria.

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